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(54) **Rainbow test device.**

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(73) Proprietor: MILES INC.
1127 Myrtle Street
Elkhart Indiana 46514(US)

(72) Inventor: Albarella, James P.
25845 Measow Oak Lane
Elkhart Indiana 46514(US)
Inventor: Charlton, Steven C.
1533 Strong Avenue
Elkhart Indiana 46514(US)
Inventor: Reinsch, James W.
3421 Whitfield Court-D
Elkhart Indiana 46514(US)
Inventor: Warchal, Mary Ellen
10272 Jefferson Road
Osceola Indiana 46561(US)

(74) Representative: Jesse, Ralf-Rüdiger, Dr. et al
Bayer AG Konzernverwaltung RP Patentabteilung
W-5090 Leverkusen 1 Bayerwerk (DE)

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Description

I. Field of the Invention

The invention relates to test compositions and test devices capable of generating different hues at different analyte concentrations. Visual tests for clinical analytes are the focus of the invention.

In particular, the invention provides a self-indicating test device for the determination of analytes, e.g. glucose, in body fluid which exhibits the colors of the RAINBOW.

II. Utility

Colorimetric tests are conveniently used as visual tests with which relatively untrained personnel can routinely obtain results by simple comparison to an appropriate color chart. Visual tests are low cost and convenient since no instrumentation is required. Presently, visual tests are used for routine screening of urine samples for a number of diagnostically important analytes, used by diabetics for home testing of urine or blood glucose and used in other fields, for example water testing for iron content.

However, currently available tests relate the intensity of a particular color to the concentration of analyte. For example, a test device may change from colorless to light blue to darker shades of blue with increasing concentration of glucose. Greater visual discrimination, and therefore greater accuracy, is possible, when a range of colors is provided rather than different shades of a single color. Therefore a test composition which exhibits different colors at different analyte concentrations would be easier to use and would provide more accurate visual results.

In order to allow visual differentiation of higher concentrations of glucose, many currently available products resort to the use of two reagent pads, one of which provides better color differentiation at the higher concentration range. Otherwise such products would exhibit only very slightly differing shades of dark blue (or dark green) above 150 mg/dL glucose. In contrast, a test device of this invention can produce dramatic color changes, blue to sea green to light yellow to red, over a range of 0 to 800 mg/dL.

This invention provides compositions which can be used to produce a range of colors, especially for clinically important analytes. In particular, a test device, capable of generating the hues of a full spectrum RAINBOW, for the determination of glucose in a body fluid, such as whole blood, is shown.

III. Information Disclosure

U.S. Patent No. 4,490,465 discloses a test system for the determination of glucose having an extended range of measurement. The system involves at least one pyridine linked dehydrogenase and one nonpyridine linked dehydrogenase. One example shows a determination of glucose with a coupled system of glucose dehydrogenase/nicotine adenine dinucleotide/diaphorase/tetrazolium salt and glucose dehydrogenase/dichlorophenolindophenol.

DE 32 11 167 which is the priority filing of US Patent 4,490,465 claims at least two enzyme systems each of which is independently capable of catalyzing the direct or indirect conversion of a substrate. The specification defines "independent of one another" to mean that, in the simultaneous presence of the systems that react with the substrate, the reaction through the second system takes place only after the coenzyme of the first system has been largely consumed.

DE 32 47 894 discloses a test system and method for the determination of reduced nicotine adenine dinucleotide, which produces an enlarged measuring range for the determination of NAD(P)H or substrates or enzymes reacting under the formation or consumption of NAD(P)H. The system is characterized in that it contains simultaneously several substances with different electrochemical potentials, functioning independently of one another as electron acceptors for NAD(P)H.

In that context, independent denotes that,for example, the reaction of the second redox indicator (having the lower electrochemical potential) only takes place after the reaction of the first (having the higher electrochemical potential) is substantially completed. Particular examples of electron acceptors include dichlorophenolindophenol and INT, (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride). The specification states that the test system can be impregnated into absorbent materials. A yellow component is added to the color seen by incorporation of a background dye, titanium yellow.

Japanese Patent Application 59-106299 was published June 19, 1984. The application discloses a method for estimating NAD(P)H with oxidized glutathione in the presence of glutathione reductase and a color forming agent. Examples of color forming agents given are 5,5'-dithiobis(2-nitrobenzoic acid), N-(1-anilinonaphthyl-4)maleimide, Beta-hydroxyethyl-2,4-dinitrophenyldisulfide, 2,2-dithiopyridine and ben-

zimidazolyl maleimide.

European Patent Application 0-153-872 discloses a method for the determination of the reduced form of nicotinamide adenine dinucleotide (phosphate) which involves reacting NAD(P)H with (1) peroxidase or thiol oxide reductase and (2) diaphorase or an electron carrier in the presence of a chromogen and determining the pigment thus formed. These two reactions do not act on a common substrate.

None of these disclosures provides a system composed of two independent catalytic systems, one being a disulfide reductase system, which allows the production of a full spectrum RAINBOW, including a yellow component generated *in situ*; the particular final hue produced depending on the concentration of the analyte.

IV. Summary of the Invention

The invention provides a test composition for the visual determination of the concentration of an analyte in a fluid sample, comprising:

(a) a catalytic system capable of generating reduced nicotinamide adenine dinucleotide by reaction with the analyte of interest; (b) a first independent catalytic system capable of generating a reduced first indicator by reaction with reduced nicotinamide adenine dinucleotide; and (c) a second independent catalytic system capable of generating a change of hue of a second indicator component by reaction with reduced nicotinamide adenine dinucleotide, which second independent catalytic system includes

(i) a disulfide reductase;

(ii) a disulfide substrate; and

(iii)a thiol indicator, wherein the disulfide reductase is capable of catalyzing the reaction between reduced nicotinamide adenine dinucleotide and the disulfide substrate to produce a product which can interact with the thiol indicator to produce the change of hue of the second indicator; whereby, the generation of the reduced first indicator and the changed hue of the second indicator occur substantially simultaneously to provide different hues at different analyte concentrations the particular final hue produced by the test composition depending on the concentration of the analyte.

Preferably one system produces a yellow hue. In one preferred system, the thiol indicator is reduced to produce a yellow second indicator.

The composition can be dissolved to provide a test solution or incorporated in a carrier matrix to provide a test device format.

A preferred embodiment is a glucose whole blood test device which is capable of generating a full spectrum rainbow, including a yellow hue generated *in situ*, the particular final hue of the test device depending on the concentration of glucose in the test sample.

V. Description of the Invention

Visual color matching is convenient and it can provide an acceptably accurate determination of analyte concentration without the need for expensive instrumentation. Color can be broken into components such as saturation, lightness and hue. Hue is commonly referred to as "color" e.g., whether something "looks" blue, red or yellow. Throughout the specification, the term "hue" is used.

Generally, one hue is associated with one form of a single indicator. Therefore in order to generate a range of hues, a number of different indicator molecules are required. The possible changes of hue are numerous. An indicator can change from one hue to another, from one hue to colorless or from colorless to a hue. It is even possible that the indicator itself will not change hue but that the format of a device incorporating the test composition is such that there is an apparent change in hue of the device when contacted with a test sample containing the analyte. For example, the hue of the indicator could not be seen prior to the reaction of the test composition with the analyte but is visible after that reaction occurs. Because it is desirable that the change of final hue exhibited by the test device from one analyte concentration to another be as clear to the user as possible, the changes colorless to a hue and a hue to colorless have been preferred. When using two or more indicators, at least one indicator component is preferably changed from one hue to colorless. Otherwise the final hue of the test composition or device will move toward black as more hues are produced.

It is particularly desirable to control the production and/or disappearance of indicators which in one form exhibit one of the primary hues: red, yellow, blue. While a composition containing two indicator components can be used, the use of three indicator components has been found to be advantageous.

For example, consider a test composition containing indicators which exhibit the following change in hue in the order shown.

indicator 1) blue to colorless
indicator 2) colorless to yellow
indicator 3) colorless to red

If the indicators react in sequence, the apparent hue of the test composition would be blue to colorless to orange (yellow + red).

However, if indicator 2 changed hue concurrently with the change in hue of indicator 1, the change in hue of indicator 3 occuring later, the apparent hue of the test composition would be blue to green (blue + yellow) to yellow to orange (yellow + red) to red. A similar example is:

indicator 1) red to colorless
indicator 2) colorless to yellow
indicator 3) colorless to blue

If the indicators reacted in sequence, the apparent hue of the test composition would be red to colorless to yellow to green (blue + yellow). If the indicators reacted as described above, with two indicator changes being produced simultaneously, the third change being delayed, the apparent hue of the test composition would be red to orange (red + yellow) to yellow to green (yellow + blue) to blue.

Both of these schemes, containing simultaneous hue changes, could produce a full spectrum RAINBOW. Such a RAINBOW would be desirable because it would provide the widest range of color visible to the eye which could be used to make the differentiation between levels of analyte easier to interpret.

The problem is twofold: 1) to select the indicators having changes in hue induced independently to give the maximum change in hue, and 2) to ensure that these indicators react in an orderly fashion depending only on the concentration of the analyte.

It has been found that a range of hues can be produced by using two independent catalytic systems, each reactive with reduced nicotinamide adenine dinucleotide (NADH) to produce one or more changes in hue. The systems react substantially simultaneously with NADH. The hue produced by a particular analyte concentration can be controlled by the relative increase or decrease of the concentrations of the components and catalysts in the test composition.

Each independent catalytic system is a system capable of interacting with one or more indicators in the presence of NADH to produce a change in hue of the indicator(s). One system contains at least an oxidized indicator and a catalyst capable of facilitating the reduction of the oxidized indicator in the presence of NADH. The other system contains at least a disulfide substrate, a thiol indicator and a disulfide reductase (the catalyst). The disulfide reductase system can function in three general ways: 1) the thiol indicator can interact with the product formed by the reaction of NADH and the disulfide substrate to produce the change of hue of the second indicator; 2) the thiol indicator can be reduced to form a reduced second indicator; and 3) that second indicator, in either case, can have a yellow hue. It has proven to be particularly difficult to generate a yellow hue *in situ*.

The terms catalyst and catalytic are used in their conventional sense herein. A "catalytic" reaction is a reaction in which the rate is changed by the addition of a "catalyst" but the catalyst itself is unchanged. The catalytic reactions referred to herein are usually enzymatic, but can also include nonenzymatic reactions such as those catalyzed with phenazine methosulfate. The term "independent" means that the oxidized indicator(s) being reduced in one catalytic system is not in equilibrium with the oxidized indicator(s) being reduced in the other catalytic system during the reaction time involved.

The NADH is generated from the analyte of interest by a catalytic system which is usually enzymatic. Table 1 shows analytes of clinical interest and useful enzymatic systems which can produce NADH. These reactions and the reaction components required are well known and are presently the basis for many diagnostic reactions which generate changes in intensity of a single hue in response to analyte concentration. The enzyme system capable of generating NADH from the analyte of interest is commonly a dehydrogenase system, although any system capable of generating NADH can be used.

TABLE 1

analyte	enzyme
glucose	glucose dehydrogenase hexokinase/glucose-6-phosphate dehydrogenase
cholesterol alcohol lactate	cholesterol dehydrogenase alcohol dehydrogenase lactate dehydrogenase

The reduced form of nicotinamide adenine dinucleotide, NADH, has been found to be a particularly useful common substrate. Although this specification refers exclusively to nicotinamide adenine dinucleotide, NAD, and its reduced form NADH, it is to be understood that the disclosure applies equally to the phosphorylated forms NAD(P) and NAD(P)H.

Catalytic systems useful for the common substrate NADH, based on diaphorase or catalysts having diaphorase like activity, such as phenazine methosulphate and 1-methoxyphenazine methosulphate, will now be described in detail.

The diaphorase system has been found to be useful as one pathway because two oxidized indicators are readily available which exhibit distinct changes in hue. DCIP, 2,6-dichloroindophenol, is a blue compound which is reduced to a colorless form in the presence of diaphorase and NADH. INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride, is colorless in the oxidized form but becomes red when reduced in the presence of diaphorase and NADH. The two changes in hue are sequential. Other indicators reactive with diaphorase and NADH can be used. For instance, N-(2,3-dimethyl-5-oxo-1-phenyl-3-pyrazolin-4-yl)- 2-chloro-6-sulfo-4-iminobenzoquinone, referred to for convenience as TR-1, and p-nitroblue tetrazolium chloride, referred to herein as NBT, can be substituted for DCIP and INT. Other indophenols and related substituted alkyl, nitro, halogen and pseudo-halogen derivatives can be substituted for DCIP, as well as other indicators of similar reduction potential capable of being reduced in the presence of NADH. Other tetrazoliums can be used in place of INT. Many are known in the art and have been catalogued in reviews such as "An Introduction to the Use of Tetrazolium Salts in Quantitative Enzyme Chemistry", F.P. Altman, Koch-light Laboratories, Ltd., Colnbrook, England, 1972; and "The Chemistry of Formazans and Tetrazolium Salts", A.W. Nineham, Chem. Rev., 55:355 (1955).

A second parallel pathway is provided by the second independent catalytic system based on a disulfide reductase. A number of reductase systems are shown in Table 2 below.

TABLE 2

disulfide substrate	disulfide reductase
L-cystine	cystine reductase
oxidized glutathione	glutathione reductase
lipoamide	dihydrolipoamide reductase (referred to as lipoamide dehydrogenase herein)
protein-disulfide	protein-disulfide reductase
oxidized thioredoxin	thioredoxin reductase
CoAS-Sglutathione	CoAS-Sglutathione reductase
asparagusate	asparagusate reductase

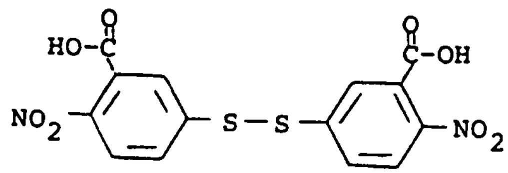
The disulfide reductase system has three components: a disulfide substrate, a disulfide reductase capable of facilitating the reaction between the disulfide substrate and NADH, and a thiol indicator which can interact with the product of the reaction between the disulfide substrate and NADH to produce the change of hue of the second indicator. A preferred disulfide substrate is lipoamide and analogs thereof which can be used with lipoamide dehydrogenase. The disulfide reductase system has been found to be particularly useful for introducing yellow into the range of hues generated by the test composition.

The thiol indicator is any substance which will interact with a thiol (-SH compound) to give observable color. Preferred thiol indicators are colorless indicators which become yellow upon interaction. Commonly, the thiol indicator is an oxidized indicator which is reduced in the presence of the product of the reaction between the disulfide substrate and NADH to produce a reduced second indicator which can be, but need not be, yellow. However, other types of interaction which will produce color are also contemplated. For

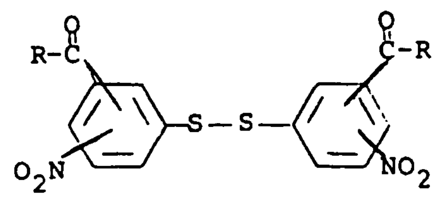
example, thiol indicators can be chelating agents such as nitroprusside, which would interact with the product of the disulfide substrate/NADH reaction to produce a red hue. A palladium complex can also be used to generate red. A yellow hue can be generated by interaction of the thiol indicator with the product of the disulfide substrate/NADH reaction by alkylation if the product is cysteine. Alternatively a cysteine product could be reacted with noradrenochrome to produce a yellow hue.

Another general type of thiol indicator is a chromophore of the desired hue, preferably yellow, immobilized behind an opaque barrier. On reaction with the product of the disulfide substrate/NADH reaction, the chromophore is released from its attachment and is free to diffuse through the opaque barrier to a position where it is visible to an observer.

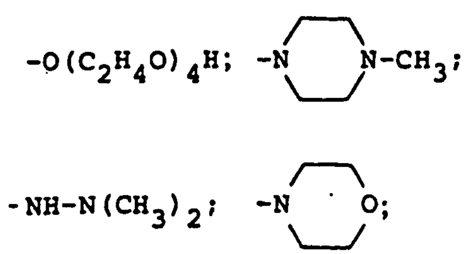
Commonly, the thiol indicators which undergo reduction are disulfide compounds. Especially preferred are analogs of 5,5'-dithiobis-(2-nitrobenzoic acid) referred to herein as DTNB, whose structure is shown below:



DTNB is commonly referred to as Ellman's Reagent. Changes at the carboxylic acid hydroxyl group have been found to be useful. Analogs of DTNB, which are defined herein to include positional isomers of DTNB, of the structure shown below are preferred:

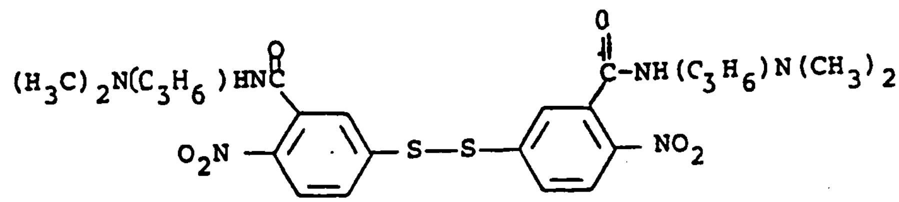


R can be many groups such as those providing an ester or amide linkage, e.g.,



While water solubilizing groups such as those shown above are preferred in gelatin matrix formats, water insoluble analogs can be used in compartmentalized formats, described later in the specification.

A preferred water soluble analog compound is 3-N-(3-dimethylaminopropyl)carboxamido-4-nitrophenyl disulfide, structure shown below, whose synthesis is detailed in the examples.



The compound is referred to subsequently in the specification as 3-ND for convenience. This compound is colorless in the oxidized form and becomes yellow in the presence of lipoamide, NADH and lipoamide dehydrogenase.

Hues of particularly useful indicators are shown in Table 3 below.

TABLE 3

	oxidized	reduced
DCIP	blue	colorless
INT	colorless	red
TR-1	red	colorless
NBT	colorless	blue
DTNB	colorless	yellow
3-ND	colorless	yellow
EA1	colorless	yellow

It has been found that a color retardant can be added to the composition. The color retardant has essentially no affect on the hue visible to the user, but its inclusion can delay the catalytic affect of the independent systems, delaying the reduction of the oxidized indicators and therefore changing the final hue produced for a particular concentration of analyte. Useful color retardants include potassium ferricyanide and 1-N-ethyl-4-methylquinolinum iodide and analogs thereof or mixtures of these compounds, potassium ferricyanide being preferred. Color retardants effectively change the measurement range of the composition.

Other components such as buffers, surfactants and polymers can be added to the composition. The pH is generally chosen to give good performance and stability to the reagents and is controlled by use of buffers. The use of buffers is preferred since the enzymes function better within the pH range of about 6.0 to 8. Choice of a buffer is within the skill of the art. Useful buffers include, but are not limited to, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 2-[tris(hydroxymethyl)methyl]amino ethanesulfonic acid (TES), 2-[N-morpholino]-ethanesulfonic acid (MES) and [3-(N-morpholino)propanesulfonic acid] (MOPS). Surfactants and polymers can be particularly useful in formulations containing a cationic tetrazolium salt as an oxidized indicator since surfactants such as polyoxyethylene ether, available under the trademark TRITON® X-100 from Sigma Chemical Co., and polymers such as polyvinyl-alcohol and polyvinylpyrrolidone, available as PVP K30 from Aldrich Chemical Co., appear to help solubilize the cationic indicator and prevent interaction with the other indicators in the test composition. Surfactants also improve wettability of the device in a dry phase formulation. Enzyme stabilizers, such as bovine serum albumin, can also be added.

Test compositions of this invention can be used by dissolving the composition in a solution or they can be incorporated into a carrier matrix and affixed to a support member such as a polyester strip to provide dry reagent strips which are well known in diagnostics. These strips provide a format which is convenient to carry and store and which is particularly useful to home users such a diabetics. Preferred compositions of this invention generate a final hue, which can be associated with a particular analyte concentration, in less than about ten minutes.

The carrier matrix employed can be any of several known in the industry, as long as the matrix can be incorporated with the composition and it does not interfere with the reactions required for the production of color. These include paper and films such as those made from natural polymers, latexes, polyurethanes, silicones or combinations of these.

In order to obtain the clearest colors possible, a clear carrier matrix was preferred. Since common analytes for this invention are water soluble compounds such as those found in body fluids, carrier matrices which can contain water, such as hydrophilic carriers, are preferred. Suitable hydrophilic carriers include

agarose, gelatin, poly(vinyl)alcohol, poly(propylimine), carrageenan and alginic acid. Other carriers could be used. Mixed multilayer carriers composed of an absorbent opaque matrix, such as paper, and a hydrophilic (e.g., gelatin) carrier layer can be advantageously used when the test components are compartmentalized.

In a preferred embodiment, a solution of 1.25% carbodiimide was used to crosslink a multilayer gelatin carrier matrix . This provides a format suitable for a whole blood glucose test which allows the blood sample to be removed from the test device by wiping the surface. Other coating materials, well known in the art, can be used to allow the device to be washed or wiped to remove a colored sample if necessary.

The hydrophilic carrier layer or layers are coated onto a rigid backing or support member such as polystyrene, polyester and the like. The backing can be opaque or transparent, although an opaque white backing is commonly preferred for visually read tests.

The number and types of components, which can be used in the independent catalytic systems described previously, is increased by the use of compartmentalization of possibly incompatible components in a test device format. Compartmentalization can take on many forms. Components can be separated by placing some in a separate layer, by solubilizing within one phase of an emulsion, by precipitation, by encapsulation and so forth. Some of the available methods are described in detail in the Examples.

A multilayer gelatin carrier was preferred for compartmentalization. INT could be placed in one layer away from the other indicator components. It was also found that the position of the components in various layers could change the hue visible to the user at a particular analyte concentration and therefore afforded another means of controlling the hue generated. The apparent hue of the device can be changed by changing the order or thickness of layering.

A particular example showing the affect of compartmentalization of components in different layers on the hue visible to the user will now be described in detail.

A RAINBOW test device for the determination of glucose can be prepared with the following independent catalytic reactions:

NADH generating system:
glucose (analyte)
glucose dehydrogenase (enzyme)
NAD (additional component)
first independent catalytic system
diaphorase (catalyst)
DCIP (oxidized first indicator, blue)
INT (oxidized third indicator, colorless)

DCIP was reduced by NADH first. Therefore the first independent catalytic system produces a blue to colorless, then a colorless to red, change in the hue visible to the user.

Second independent catalytic system:
lipoamide (disulfide substrate)
lipoamide dehydrogenase (disulfide reductase)
DTNB (thiol indicator, oxidized second indicator, colorless)

Compartmentalization of the oxidized indicators, e.g., placing the indicators in different gelatin layers, can effectively change the hue seen at different glucose concentrations even though the concentrations of test components are the same. As an example, three films were made with the indicators arranged in different layers, but keeping the concentrations of the components of the total test composition the same.

FILM A

DCIP, DTNB, Enzymes
INT

FILM B

INT
DCIP, DTNB, Enzyme

FILM C

Enzyme
INT
DCIP, DTNB

In gelatin, certain water soluble molecules, such as DNTB, are capable of diffusing through the gelatin after contact with an aqueous sample, while others, such as INT, do not readily migrate.

In Film A, glucose reacts with the enzymes, then the NADH produced reacts with the independent catalytic systems to reduce DCIP and DTNB. However, reduction of INT is delayed until the NADH can diffuse into the lower layer of the film. At 250 mg/dL glucose, Film A is yellow orange.

In Film B, the glucose must diffuse through the INT layer before reaching the enzymes to react and produce NADH. With the production of NADH, the DCIP will be reduced but the reduction of INT is delayed since the NADH must diffuse back up onto the top layer before the INT can be reduced. Therefore, at 250 mg/dL glucose, Film B is yellow-green. The compartmentalization of INT above the enzymes and other indicator components has changed the color observed at this particular concentration of glucose.

In Film C, the glucose reacts with the enzymes in the top layer. As the NADH migrates through the INT layer, a small amount of INT reduced causing a slight red color. As the NADH migrates through to the bottom layer, the DCIP and the DTNB are reduced. Finally, since not all the NADH has reacted due to the concentration of components chosen, the NADH will react with the INT. At 250 mg/dL glucose, Film C is a reddish orange.

The ultimate goal of the invention was to generate distinctly different hues at different analyte concentrations. It has been shown that this goal can be achieved with test compositions of this invention with a variety of methods as summarized below.

1) Choose oxidized indicators which will generate the desired hue or turn colorless upon reduction.

2) Choose indicators for reaction in a particular catalytic system which have differing reduction potentials which will control the sequence of reactions in that system.

3) Control the quantities of components in the independent catalytic systems so the components involved in one reaction sequence are essentially exhausted at chosen analyte concentrations.

4) Increase (or decrease) the amount of catalyst in a system. This will increase (or decrease) the rate of interaction or reduction of the indicator components by that system and therefore change the apparent hue at a particular analyte concentration.

5) Compartmentalize the components of the reaction system in a test device. The advantages of compartmentalization can include the ability to: change the apparent final hue of the device even when component concentrations are the same; utilize incompatible indicators or water insoluble indicators; and utilize enzyme systems normally inhibited by thiol indicators.

6) Add a color retardant which will delay the reactions of the independent catalytic systems.

The methods suggested above can be combined.

The invention will now be illustrated, but is not intended to be limited, by the following examples:

VI. Examples

Abbreviations

MPMS	1-methoxyphenazine methosulphate
PMS	phenazine methosulphate

DCIP	2,6-dichloroindophenol
TR-1	N-(2,3-dimethyl-5-oxo-1-phenyl-3-pyrazolin-4-yl)-2-chloro-6-sulfo-4-iminobenzoquinone (for preparation see Example 1A)
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
NBT	*p*-nitroblue tetrazolium chloride
DTNB	5,5-dithiobis(2-nitrobenzoic acid)
3-ND	3-N-(3-dimethylamino propyl) carboxamido-4-nitrophenyl disulfide (for preparation see Example 1B)
EA1	Dibutyl-5,5'-dithiobis-(2-nitrobenzoate (for preparation see Example 2C)
NAD^+	Nicotinamide-adenine dinucleotide, lithium salt
HEPES	buffer, N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid
MES	buffer, 2-[N-morpholino)-ethanesulfonic acid
TAPSO	buffer, 3-(N-tris(hydroxymethyl)methylamino)-2-hydroxypropane-sulfonic acid
TRIS	buffer, Tris(hydroxymethyl)-aminomethane
Triton® X-100	surfactant, polyoxyethylene ether available from Sigma Chemical Co.
GDH	glucose dehydrogenase (EC 1.1.147) capable of producing NADH
LipDH	lipoamide dehydrogenase
LDH	lactate dehydrogenase
U	International Units, a measure of enzyme activity (one U is the enzyme activity required to catalyze the conversion of one micromole of substrate per minute under specified conditions of temperature and pH
PET	polyethylene terephthalate
FMN	flavin mononucleotide
PE 310	polyethylene coated paper
BSA	Bovine Serum Albumin
PVP K30	polyvinylpyrrolidone, molecular weight 40,000, available from Aldrich Chemical Co.
dL	deciliters
mL	milliliters
µL	microliters
g	grams
mm Hg	millimeters of mercury, pressure designation
mp	melting point
µ	microns
RT	room temperature, usually 25 • C

Example 1: Preparation of Compounds

A. N-(2,3-dimethyl-5-oxo-1-phenyl-3-pyrazolin-4-yl)-2-chloro-6-sulfo-4-iminobenzoquinone (TR-1)

TR-1 is an indicator which is red in the oxidized form and is colorless upon reduction. It has a reduction potential similar to DCIP and has been used to produce a "reverse" RAINBOW. TR-1 was prepared as follows:

Ammonium hydroxide (1N, 10mL) was added to a mixture of 0.4 g (1.9 mmol) of 4-aminoantipyrine, and 0.5 g (1.7 mmol) 2-hydroxy-3,5-dichlorobenzene sulfonic acid disodium salt in 50 mL of water. After stirring briefly, 1.3 g (3.8 mmol) of potassium ferricyanide ($K_3Fe(CN)_6$) was added and the reaction allowed to stir at room temperature for one hour. The mixture was filtered to yield 0.2 g (21%) of a dark brown solid. The product was homogeneous on thin layer chromatography (silica gel, with 4:1 chloroform/methanol) and apparently was a mixture of alkali and ammonium salts.

Analysis: Calculated for $C_{17}H_{10}ClN_3O_5Na$: C, 47.54; H, 3.04; N, 9.72.
Found: C, 46.10; H, 3.61; N, 11.00
^1H NMR (D_6DMSO) δ: 7.80-7.20 (m, 7H), 3.40 (s, 3H), 2.53 (s, 3H),
IR (KCl): 1660, 1630, 1400 cm^{-1}.

B. 3-N-(3-dimethylaminopropyl)carboxamido-4-nitrophenyl Disulfide (3-ND)

A water soluble analog of 5,5'-dithiobis-(2-nitrobenzoic acid) was prepared for use as a thiol indicator. This is a preferred indicator for use with the disulfide reductase system which is colorless in the oxidized form and becomes yellow on reduction. The compound, 3-ND, was prepared as follows:

A suspension containing 7.93 g of 3-carboxy-4-nitrophenyl disulfide (20 mmol), 1.2 mL of dry N,N-dimethylformamide (1.55 mmol), 11.7 mL of thionyl chloride (60.3 mmol) and 400 mL of dichloromethane (CH_2Cl_2) was refluxed for four hours and then was allowed to stir overnight at ambient temperature. The resulting clear solution was evaporated in vacuo (12 mm followed by 0.1 mm Hg) to a yellow solid. The residue was then placed under an argon atmosphere, dissolved in 200 mL of CH_2Cl_2, cooled to 0°, and then treated with 10.1 mL of 3-dimethylaminopropylamine (80 mmol). The reaction mixture became a dark orange and a precipitate was formed. The resulting solution was allowed to warm to ambient temperature overnight. The reaction mixture was then successively extracted four times with 200 mL portions of 5% sodium bicarbonate solution, twice with 200 mL of water, and once with brine. The organic layer was then dried over magnesium sulfate, filtered, and concentrated to give 9.39 g of a dark orange oil. The mixture was then flash chromatographed on 500 g of SiO_2-60 (70-230 mesh, available as Silica gel-60 from Merck & Co.) equilibrated and eluted with 50:10:1 dichloromethane/methanol/concentrated ammonium hydroxide solvent mixture. Fractions 110 to 205 containing the purified product were pooled and concentrated in vacuo to give 7.06 g of an orange solid. The crude product was recrystallized from ethyl acetate using a treatment with pulverized carbon black such as norite and a diatomaceous earth, a filtering aid available from Manville Products Corp., Denver, Colorado, under the trademark Celite®. Obtained was 5.47 g of light yellow crystals after drying at 55°, 0.1 mm. Yield = 48.4%. mp 152-156°.

Analysis: Calculated for $C_{24}H_{32}N_6O_6S_2$: C, 51.05; H, 5.71; N, 14.88.

Found: C, 51.05; H, 5.56; N, 14.62.

PMR (60MHz, $CDCl_3$) δ:1.73 (quintet, J=6Hz, 4H); 2.17 (s, 12H); 2.45 (t, J=6Hz, 4H); 3.53 (q, J=6Hz, 4H); 7.57 (s, 2H); 7.65 (dd, J=7Hz, 2Hz, 2H); 8.02 (m, 2H, N-H); 8.03 (d, J=7Hz, 2H).

IR(KBr) cm^{-1}: 3260, 3060, 2940, 2870, 2820, 2790, 1650, 1560, 1530, 1470, 1345.

Mass Spectrum (FAB) m/e: 565 (M+1, 13.6%).

C. Dibutyl-5,5'-Dithiobis-(2-nitrobenzoate) (EA-1)

This compound, which is referred to herein as EA-1, is a lipophilic analog of 5,5'-dithiobis-(2-nitrobenzoic acid). It was prepared for use as a thiol indicator as follows:

A suspension containing 6.85 g (17.25 mmol) of 5,5'-dithiobis-(2-nitrobenzoic acid), 3.46 mL of thionyl chloride, 0.346 mL of N,N-dimethylformamide, and 350 mL of dichloromethane were heated to reflux for 3 hours. Additional thionyl chloride (3.46 mL) and N,N'-dimethylformamide were added and refluxing was continued for 2 hours. A clear, light green solution was obtained, indicating complete conversion to the bis-acid chloride. The solvents were removed in vacuo and the resulting light-green solid was placed under an Argon atmosphere. The residue was then suspended in 100 mL of pyridine and treated with 20 mL of n-butanol. A mild exotherm and a darkened but homogeneous reaction was obtained, which was allowed to stir overnight. The reaction solvents were removed in vacuo and the residue was dissolved in 300 mL of chloroform. The organic layer was then successively extracted thrice with 200 mL of 0.1 N HCl, twice with 200 mL of 5% Na HCO_3 solution, and with 200 mL of brine. Drying ($MgSO_4$), filtration, and removal of solvent gave 12.79 g of an oil which was dissolved in ethyl acetate and adsorbed in vacuo onto a small amount of SiO_2-60. The impregnated solid was then placed atop a column of 500 g of SiO_2-60 (230-400 mesh) which had been packed and equilibrated with 8:1 hexane-ethyl acetate. The column was then flash chromatographed using this solvent mixture with fractions of 25 mL collected. Fractions 190-270 were pooled and concentrated to give 8.07 g of product as an oil which solidified upon standing (92% yield).

Analysis: Calculated for: C, 51.96; H, 4.76; N, 5.08.

Found: C, 52.49; H, 4.88; N, 5.45.

PMR (60 MHz, $CDCl_3$) δ: 0.97 (t, J=7Hz, 6H, CH_3-CH_2); 1.2-1.9 (m, 8H, -CH_2-CH_2-); 4.37 (t, J=6Hz, 4H, -O-CH_2-CH_2-) 7.77 (s, 2H, C_6H); 7.83 (AB quartet, J=8Hz, 4H, C_3H, C_4H).

IR (KBr)cm^{-1}: 1730

$$(Ar-\overset{\overset{O}{\|}}{C}-O-CH_2-\ \text{stretch}).$$

Mass Spectrum: E.I. (70 EV) m/e = 508 (37%, M^+).

D. Dimethyl 5,5'-Dithiobis-(2-nitrobenzoate)

This compound is a lipophilic analog of 5,5'-dithiobis-(2-nitrobenzoic acid), which was prepared for use as a thiol indicator as follows:

A suspension containing 7.93 g (20 mmol) of 5,5'-dithiobis-(2-nitrobenzoic acid), 1.2 mL of N,N-dimethylformamide, 11.7 mL of thionyl chloride, and 400 mL of dichloromethane were refluxed for 4 hours until a clear light green solution was obtained. The solvents were then evaporated in vacuo to obtain a yellow solid which was then placed under an Argon atmosphere, dissolved in 200 mL of dichloromethane, and cooled to 0°. The stirred mixture was then treated with 4.03 mL of dry pyridine (50 mmol) and 30 mL of methanol. The resulting mixture was then allowed to warm to ambient temperature overnight. The reaction mixture was then successively extracted thrice with 300 mL of 5% $NaHCO_3$ solution, thrice with 300 mL of 1 M citric acid, and 300 mL of brine. Drying ($MgSO_4$), filtration and removal of solvents gave 8.29 of a yellow solid which was recrystallized in two crops from toluene as a light yellow solid in 92% yield. mp 103-104.5°.

Analysis: Calculated for: C, 45.28; H, 2.86; N, 6.60.

Found: C, 45.74; H, 3.01; N, 6.25.

PMR (60 MHz, $CDCl_3$) δ: 3.93 (s, 6H, -0-CH_3); 7.8 (s, 2H, C_6H); 7.83 (AB quartet, J=8Hz, 4H, C_3H, C_4H).

IR (KRr) cm^{-1} : 1740

$$(-\overset{\overset{\displaystyle O}{\|}}{C}-O-CH_3 \text{ stretch}).$$

Mass Spectrum: EI (70EV) m/e: 424.3 (M^+, 67.7%).

E. 3,6-Dioxaoctyl-5,5'-Dithio-(2-nitrobenzoate)

This compound is a water soluble analog of 5,5'-dithiobis-(2-nitrobenzoic acid). It was prepared for use as a thiol indicator as follows:

A suspension containing 7.93 g (10 mmol) of 5,5'-dithio-(2-nitrobenzoic acid), 1.17 g of N,N-dimethylformamide, 11.7 mL of thionyl chloride, and 400 mL of dichloromethane was heated to reflux with stirring for 2 hours to obtain a clear solution. The solvents were removed in vacuo to give a light green solid which was placed under Argon, cooled to 0°, and suspended with stirring in 120 mL of dry pyridine. Carbitol® (20 mL) was then added and the resulting mixture became an orangish-red homogeneous solution after 1 hour reaction time (Carbitol® is a registered trademark of Dow Chemical Co. and is chemically named 2-(2-ethoxyethoxy)ethanol). The mixture was then allowed to come to ambient temperature overnight. The sample was then evaporated in vacuo to a dark oil and flash chromatographed on 500 g of SiO_2-60 (230-400 mesh) packed and eluted with a 0.5% methanol-chloroform solvent mixture. Fractions of 25 mL were collected. Fractions 150-186 were pooled and concentrated to give 7.86 g of a yellow oil. The sample was then dissolved in ether, treated with 4 g of Norit, filtered through Celite, and precipitated as a dense yellow oil with hexane. The solvents were then decanted and the residual solvents were removed in vacuo at 40° (0.1 mm) for 1 hour to give 5.48 g of a viscous, yellow oil (44% yield).

Analysis: Calculated for: C, 49.67; H, 5.13; N, 4.46.

Found: C, 49.41; H, 5.09; N, 4.37.

PMR (60 MHz, $CDCl_3$) δ: 1.2 (t, J=7Hz, 6H, $\underline{CH_3}$-CH_2-O-); 3.5 (q, J=7Hz, 4H, CH_3-$\underline{CH_2}$-O-); 3.6 (s, 8H, -CH_2-CH_2-O-); 3.8

$$(m,\ 4H, -\overset{\overset{\displaystyle O}{\|}}{C}-O-CH_2-\underline{CH_2}-);$$

4.55

$$(\mathrm{m},\ 4\mathrm{H},\ -\overset{\overset{\displaystyle O}{\|}}{C}-\underline{CH_2}-CH_2-O-);$$

7.8 (s, 2H, C_6H); 7.82 (AB quartet, J = 8Hz, 4H, C_3H, C_4H).
IR ($CHCl_3$)cm^{-1} : 1740

$$(-\overset{\overset{\displaystyle O}{\|}}{C}-O-CH_2-\ \mathrm{stretch}).$$

F. 3,6,9,12-Tetraoxadodecyl 5,5'-Dithio-(2-Nitrobenzoate) and 1,12-Cyclic Diester

Both of these compound are water soluble analogs of 5,5'-dithiobis-(2-nitrobenzoic acid). They were prepared for use as thiol indicators as follows:

A suspension containing 7.93 g (20 mmol) of 5,5'-Dithiobis-(2-nitrobenzoic acid), 11.7 ml of thionyl chloride, and 1.2 mL of N,N-dimethylformamide was heated to reflux for 2 hours to obtain a clear yellow solution. The solvents were removed in vacuo to give a light green solid, which was dissolved in a mixture of 50 mL of dichloromethane and 5 mL of pyridine. This solution was then added slowly to a 0° solution of 34.5 mL (38.8 g, 200 mmol) of tetraethylene glycol in 100 mL of dichloromethane under an Argon atmosphere. The reaction mixture was allowed to come to ambient temperature overnight. Evaporation of solvents in vacuo gave a brown oil which was partitioned between chloroform and water. The aqueous layer was extracted again with chloroform and the combined organic layers were washed with brine and dried (Na_2SO_4). Filtration and evaporation of solvent in vacuo gave an orange oil which was flash chromatographed on 500 g of SiO_2-60 (230-400 mesh) packed and eluted with a 5% methanol-chloroform solvent mixture. Fractions of 25 mL were collected. Fractions 24-38 were pooled and concentrated to give 2.21 g of a yellow glass. This was identified as the 1,12-cyclic-3,6,9,12-tetraoxaundecyl ester of 5,5'-dithiobis-(2-nitrobenzoic acid). The yield of cyclic ester was 20%.
^{13}C NMR (22.5 MHz, $CDCl_3$) δ: 66.8, 68.3, 70.4 (all -O-CH_2-CH_2-O-); 128.8, 142.4, 146.3 (aryl C); 164.3

$$(-\overset{\overset{\displaystyle O}{\|}}{\underline{C}}-O-CH_2).$$

IR ($CHCl_3$)cm^{-1} : 1730 cm^{-1}

$$(-\overset{\overset{\displaystyle O}{\|}}{C}-O-CH_2-\ \mathrm{stretch}).$$

Fractions 91-101 were pooled and concentrated to give 3.94 g of the expected 3,6,9,12-tetraoxadodecyl diester. (26% yield).
^{13}C NMR (22.5 MHz, $CDC1_3$) δ: 61.6, 65.6, 68.3, 70.2, 70.5, 72.4 (all -O-CH_2-CH_2-O-); 129.0, 142.4, 146.5 (aryl C); 164.5

$$(-\underline{\overset{\overset{O}{\|}}{C}}-O-CH_2-).$$

PMR (60 MHz, $CDCl_3$): integration ratio of aliphatic to aromatic protons = 5.6.
IR ($CHCl_3$)cm^{-1} : 3600-3350 (-CH_2-$\underline{OH}$ stretch): 1730

$$(-\overset{\overset{O}{\|}}{C}-)-CH_2-\text{ stretch}).$$

Example 2: Glucose Formulations

Glucose test devices useful for testing whole blood can be prepared as follows. Reduced nicotinamide adenine dinucleotide was the common substrate and was generated from glucose by glucose dehydrogenase. The general chemistry for RAINBOW glucose formulations is shown schematically on a following page. The thiol indicator, DTNB, is commonly called Ellman's Reagent.

A. Pathway 1: Diaphorase/NBT/TR-1:
Pathway 2: LipDH/lipoamide/DTNB

Layer	Component	Quantity (g)
1	Gelatin	1.13
	Water	6.78
	PVP K30 (20%)	1.69
	Triton® X-100 (4%)	0.40
	NBT	0.064
2	Gelatin	1.13
	HEPES Buffer, 1 M, pH 7.5	5.42
	Water	1.36
	PVP K30 (20%)	1.69
	$K_3Fe(CN)_6$	0.073
	DTNB	0.040
	GDH (64.6 U/mg)	0.060
	Diaphorase (118 U/mg)	0.005
	BSA	0.035
	Lipoamide	0.030
	LipDH (1350 U/mL)	200 µL
	NAD^+	0.060
	Mutarotase (5060 U/mL)	100 µL
	Triton® X-100 (4%)	0.4
3	Gelatin	1.13
	Water	6.78
	Triton® X-100 (4%)	0.40
	TR-1	0.060
4	carbodiimide	1.25%

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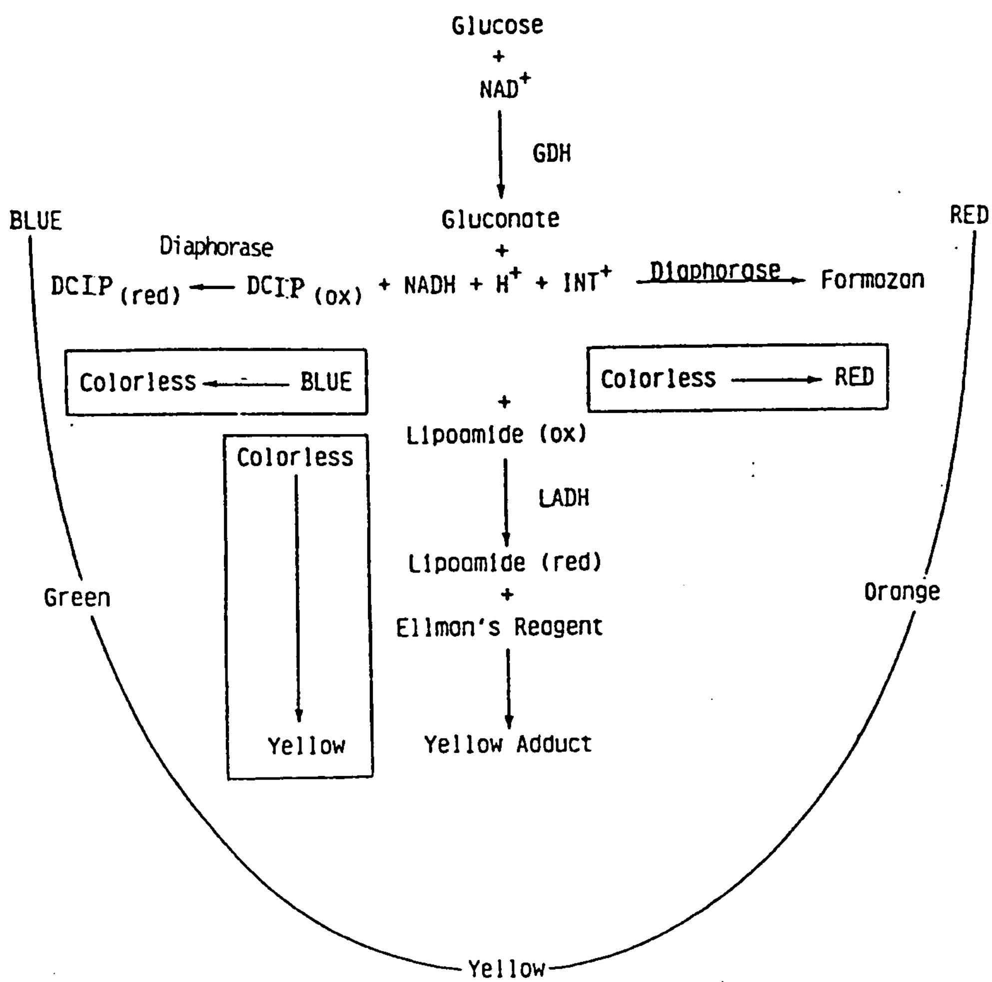

Procedure: The components of each solution were combined at 40° in the order given. The solutions were degassed before coating. Layer 1 was spread onto a polyester backing and dried. Layer 2 was spread onto Layer 1 and dried and so on. The final device was made up of three gelatin layers on a polyester backing. The carbodiimide coating provided a hardened surface by crosslinking the gelatin, which allows wiping the surface of the device.

Dose Response: With increasing glucose concentration from 50 to 600 mg/dL, the color sequence was pale red to olive green to blue black.

B. Pathway 1: MPMS/DCIP/INT
Pathway 2: Glutathione Reductase/Glutathione/DTNB

Layer	Component	Quantity (g)
1	Gelatin	1.13
	Water	6.78
	PVP K30 (20%)	1.69
	Triton® X-100 (4%)	0.40
	INT	0.064
2	Gelatin	1.13
	HEPES Buffer, 1 M, pH 7.5	5.42
	Water	1.36
	PVP K30 (20%)	1.69
	Triton® X-100 (4%)	0.40
	DCIP	0.0147
	$K_3Fe(CN)_6$	0.073
	DTNB	0.040
	GDH (646 U/mg)	0.060
	NAD^+	0.060
	MPMS	0.010
	Glutathione Reductase (2320 U/mL)	230 µL
	Glutathione	0.089
	Mutarotase (5060 U/mL)	100 µL
3	Carbodiimide	1.25%

The procedure was used for the formulation and testing of the device that of Example 2A.

Dose Response:

Glucose (mg/dL)	Color
110	Peacock Blue
140	Green-Blue
180	Dull Light Aqua
250	Dull Rose
300	Dull Rose
400	Rose
500	Rose
600	Dark Rose
800	Burgandy

The hues, blue to rose to burgandy, can be easily distinguished visually.

C. Pathway 1: MPMS/DCIP/INT -
Pathway 2: LipDH/lipoamide/DTNB

Layer	Component	Quantity (g)
1	Gelatin	1.13
	Water	6.78
	PVP K30 (20%)	1.69
	Triton X-100 (4%)	0.40
	INT	0.064
2	Gelatin	1.13
	HEPES Buffer, 1 M, pH 7.5	5.92
	Water	1.86
	PVP K30 (20%)	1.69
	Triton X-100 (4%)	0.40
	$K_3Fe(CN)_6$	0.073
	DTNB	0.040
	GDH(64.6 U/mg)	0.060
	NAD^+	0.060
	MPMS	0.010
	DCIP	0.0147
	Lipoamide	0.030
	Mutarotase (5060 U/mL)	100 µL
	LipDH (1350 U/mL)	200 µL
3	Carbodiimide	1.25%

The procedure used for the formulation and testing of the device was that of Example 2A

Dose Response:

Glucose (mg/dL)	Color
20	Peacock Blue
40	Peacock Blue
70	Aqua
110	Teal Green
140	Mint Green
180	Sea Green
250	Green Gold
300	Gold
400	Orange Gold
500	Orange
600	Dark Orange
800	Darker Orange

The colors blue, green, gold, orange are visually distinct.

D. Pathway 1: DCIP/INT/MPMS
Pathway 2: LipDH/lipoamide/3-ND

Layer	Component	Quantity (g)
1	Gelatin	1.13
	Water	6.78
	PVP K30 (20%)	1.69
	Triton® X-100 (4%)	0.40
	INT	0.064
2	Gelatin	1.13
	HEPES Buffer, 1 M, pH 7.5	5.92
	Water	1.86
	PVP K30 (20%)	1.69
	Triton® X-100 (4%)	0.40
	$K_3Fe(CN)_6$	0.073
	DCIP	0.0147
	3-ND (20 mM)	1.25 mL
	GDH	0.060
	NAD^+	0.060
	MPMS	0.010
	Lipoamide	0.030
	Mutarotase (5060 U/mL)	100 μL
	LipDH (1350 U/mL)	200 μL
3	Carbodiimide	1.25%

The procedure used for the formulation and testing of the device was that of Example 2A.

Dose Response:

Glucose (mg/dL)	Color
40	Peacock Blue
70	Teal Green
110	Mint Green
140	Light Mint
180	Sea Green
250	Tan
300	Dark Tan
400	Pale Brown
500	Rust
600	Rust
800	Dark Red

With this formulation, using the DTNB derivative 3-ND, the generation of the final hue of the test device, visible to the user, was complete in 8 minutes.

E. Pathway 1: MPMS/DCIP/INT
Pathway 2: LipDH/lipoamide/3-ND

Layer	Component	Quantity (g)
1	gelatin	1.13
	water	6.78
	PVP K30 (20%)	1.69
	Triton® X-100 (4%)	.40
	INT	.064
2	gelatin	1.13
	HEPES Buffer, 1M ph=7.5	5.92
	water	1.86
	PVP K30 (20%)	1.69
	Triton X-100 (4%)	.40
	3-ND (40 mM)	2.50 mL
	DCIP	0.0147
	GDH	0.060
	NAD^+	0.060
	MPMS	0.010
	lipoamide	0.030
	mutarotase (5060 U/mL)	100 μL
	LipDH (1350 U/mL)	200 μL
	$K_3Fe(CN)_6$	0.055
3	carbodiimide	1.25%

Dose Response:

Glucose mg/dL	Hue generated
0	blue
20	dark blue green
40	blue green
70	light green
110	yellow green
140	gold
180	gold orange
250	orange
400	red orange
800	deep red

This device exhibits a full spectrum RAINBOW; blue, green, gold, orange red. The hue visible to the user depends on the concentration of glucose and is generated in 7 to 8 minutes.

F. Whole blood glucose RAINBOW test device.
Pathway 1: Diaphorase/DCIP/INT
Pathway 2: LipDH/lipoamide/3-ND

Layer	Component	Quantity (g)
1	Gelatin Water PVP K30 (20%) Triton® X-100 (4%) INT	1.13 6.78 1.69 .40 .064
2	Gelatin MES buffer, 1M (pH-6.5) Water PVP K30 (20%) Triton® X-100 (4%) 3-ND (40 mM) GDH (64 U/mg) NAD+ Diaphorase (118 U/mg) BSA Mutarotase (5060 U/m) LipDH (1350 U/mL) DCIP $K_3Fe(CN)_6$	1.13 5.92 1.86 1.69 .40 2.5 mL .060 .060 .010 .040 100 µL 200 µL 0.0147 0.055
3	Carbodiimide	1.25%

The procedure used for formulation of the device was that described in Example 2A. Testing was done with whole blood samples spiked to the desired glucose levels, analyzed with a YSI glucose analyzer. The dose response data is given below. The test device, containing a test composition of the invention compartmentalized by layering in a gelatin carrier, exhibited a full spectrum RAINBOW over a glucose concentration of 0 to 800 mg/dL. The composition was designed to produce a green hue covering the normal blood glucose range.

Dose Response:

Glucose mg/dL	Hue Generated
0	blue
40	light blue
70	blue green
110	sea green
140	pale green
180	light yellow
250	yellow orange
400	orange red
800	red

G. Rainbow: Varying enzyme concentration.

The following example was designed to show how the final hue produced can be controlled by changing the concentrations of the enzymes.

Pathway 1: Diaphorase/DCIP/INT

Pathway 2: LipDH/lipoamide/3-ND

Layer	Components	Quantity (g) A	B	C	D
1	Gelatin	1.13	1.13	1.13	1.13
	Water	6.78	6.78	6.78	6.78
	PVP K30® (20%)	1.69	1.69	1.69	1.69
	Triton® X-100 (4%)	.40	.40	.40	.40
	INT	.064	.064	.064	.064
2	Gelatin	1.13	1.13	1.13	1.13
	Hepes buffer, 1M pH 7.5	5.92	5.92	5.92	5.92
	Water	1.86	1.86	1.86	1.86
	PVP K30® (20%)	1.69	1.69	1.69	1.69
	Triton® X-100 (4%)	.40	.40	.40	.40
	3-ND (40 mM)	2.5 m	2.5 mL	2.5 mL	2.5 mL
	DCIP	0.0147	0.0147	0.0147	0.0147
	GDH	.060	.060	.060	.060
	NAD+	.060	.060	.060	.060
	Diaphorase(118 U/mg)	.010	.010	.010	.025
	Lipoamide	.030	.030	.030	.030
	BSA	.040	.040	.040	.040
	Mutarotase (5060 U/mL)	100 µL	100 µL	100 µL	100 µL
	LipDH (1350 U/ml)	50 µL	200 µL	800 µL	200 µL
3	Carbodiimide	1.25%	1.25%	1.25%	1.25%

The films were prepared and tested as described in example 2A. The results are shown in the following table.

Glucose mg/dL	A	B	C	D
0	medium blue	medium blue	medium blue	dark blue
20	medium blue	light blue	medium blue	dark blue
40	medium blue	light blue	light blue	dark blue
70	light blue	aqua	aqua	medium blue
110	aqua	light green	light green	aqua
140	light aqua	light green	yellow beige	light aqua
180	peach beige	light yellow-peach	yellow gold	greyish green blue
250	light rust	dirty gold	orange gold	dark peach
400	rust orange	rust orange	orange	burnt orange
800	dark rust orange	dark red rust	dark rust orange	deep red

Example 3: Paper RAINBOW

Although multilayered gelatin is a preferred matrix for a rainbow device, a device can be prepared using paper as a carrier. A solution of the following composition, made in the order shown was prepared.

Reagent	PVA	Triton X-100	Buffer	INT	DCIP	DTNB
Concentration (mM) Stock Solution	10	10	1000	5	5	10
Volume (µL) Used	750	100	1000	1500	900	1500
Final Concentration (mM)	1.24	0.17%	166	1.24	0.74	0.68
Order of Mixing	1	2	3	4	5	6

Reagent	MPMS	Glutathione	Glut-Reductase
Concentration (mM) Stock Solution	0.05	5	1790 U/mL
Volume (µL) Used	30	150	112
Final Concentration	250 nM	124 µM	33 U/mL
Order of Mixing	7	8	9

The buffer was TAPSO, pH 7.4. Both polyvinylalcohol (PVA) and the order of reagent addition were critical in this example. A concentration of 1 to 1.5% PVA prevents the coprecipitation of DCIP and INT in a paper matrix.

Whatman 31 ET paper was impregnated with this solution and dried at 50 °C. The dried paper was cut and mounted on a plastic backing to form test strips. The test strips were assayed with NADH solution and the results shown below.

Time (minutes)	NADH Concentration (mM)				
	0	.33	1	3.33	10
1	blue	light blue	blue/green	brown	brown/orange
2	blue	light blue	pale green	brown	orange
3	blue	light blue	faint green	brown	orange

Example 4: NADH Generating Systems Containing Enzymes Sensitive to Thiol Reagents

A general problem with the described thiol detection reagent system is that thiol indicators can react with protein systems, frequently leading to inactivation of enzyme. Many such enzyme inhibitions by DTNB are described in the literature. There are two general solutions, both involving compartmentalizing the thiol reagent: (1) sequester the thiol indicator in an organic phase or as insoluble particles; (2) physically separate the sensitive enzyme and the thiol reagent by immobilizing the latter.

A. Approach (1) - Sequestering the Reagent.

A water insoluble analog of DTNB, EA1 was used (for preparation see example 1C).

There are two approaches to sequestering the reagent: (a) EA1 was dissolved in an oil and dispersed as an emulsion in a gelatin film.

Organic Phase: EA1 (300 mM) was dissolved in tricresyl phosphate with trioctylamine (1%).

Aqueous Phase	Final Concentration
Gelatin	10%
Potassium Phosphate, pH 7.5	50 mM
DCIP	1.5 mM

The oil phase was emulsified with the aqueous phase in a Waring Blender to a final concentration of 5%. Lipoamide dehydrogenase (Sigma type III, 13.3 U/mL) was added to the emulsion. The emulsion was coated on a plastic support (170 µ) and dried at room temperature.

Treatment of the dried film with NADH/Lipoamide (approximately 1mM) at pH 8.5 (tris buffer) produced a very rapid bleaching of the blue hue of DCIP and formation of yellow by the EA1 reagent. Without NADH there was no reaction.

The enzyme lactate dehydrogenase (LDH), which is inhibited by DTNB was tested with this film. LDH, approximately 150 U/mL, and lipoamide (approximately 1mM) in about 250 mM lactate, pH 8.5, produced a yellow hue in about 10 minutes. The blank, without LDH, was uncolored. The conclusion is that the use of an oil soluble thiol indicator, if compartmentalized in an oil emulsion, permits the use of enzymes which are commonly believed to be sensitive to thiol reagents.

(b) - EA1 deposited directly into paper.

EA1 was dissolved in toluene to a concentration of 5.7 mM. Whatman 31 ET paper was impregnated with this solution and dried at 50°. A second solution was prepared of the following constituents:

	Final Concentration
Triton® X-100	0.1%
$(tris)_2$ sulphate pH 8.5	0.2 M
PVA (98.5% hydrolyzed)	0.62%
MPMS	0.25 µM
Lipoamide	0.25 mM
LipDH	30 U/mL
NAD	0.5 mM
DCIP	0.8 mM

$(Tris)_2$ sulphate is Tris buffer adjusted to the desired pH with sulfuric acid.

The dried paper was dipped into the second solution and dried again at 50°C and affixed to a plastic support.

Test solutions for LDH contained L-lactate (167 mM), $(tris)_2$ sulphate, pH 8.5, (0.33 M) and LDH enzyme (rabbit muscle, Sigma type II) as shown in the following table with the test results.

Time (minutes)	LDH Concentration (μ/mL)				
	0	0.333	0:666	1.667	3.333
3	dark blue	mid-blue	pale blue	blue/green	mustard
4	dark blue	mid-blue	very pale blue	bluish yellow	yellow

Very good color discrimination is seen between the various levels of LDH.

Test Solution for alcohol detection

The ability of this compartmentalized film format to handle assays requiring the use of an enzyme which is sensitive to thiol reagents was further tested using alcohol dehydrogenase.

Test solutions were prepared which contained various concentrations of ethanol (tris)$_2$ sulphate buffer, 0.5 M, pH 8.5, and alcohol dehydrogenase (from Baker's yeast, Sigma Catalog #A7011, 22.5 U/mL).

The test strips prepared as described above were used. The results are shown in the following table.

Time (minutes)	Ethanol Concentation (mg/dL)					
	0	1.84	3.22	4.6	6.9	11.5
3	dark blue	less blue	medium blue	pale blue	blue/green	mustard
4	dark blue	less blue	medium blue	pale blue	mustard	yellow

Very good discrimination was seen between levels of alcohol.

From these experiments it was concluded that the RAINBOW system utilizing a thiol detection reagent can be used with thiol reagent sensitive enzymes.

B. Approach 2: Physical separation of thiol reagent sensitive enzyme and thiol reagent by immobilizing the latter.

DTNB can be covalently bound to a large molecule or can be physically trapped in a matrix, such as gelatin, which only allows penetration of small molecules. Therefore direct interaction between the enzyme and thiol reagent would not be expected.

Preparation of immobilized DTNB

DTNB was linked to human serum albumin through the carboxyl function using a water soluble carbodiimide reagent.

A 30% human serum albumin solution was made 0.2M in sodium phosphate and the pH adjusted to 4.57. It was then diluted to 4.8% albumin. DTNB (10.7 mM) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, 10.7 mM) were dissolved in a small amount of ethanol and added, with vigorous stirring, to the albumin solution maintained at 0 °C. After 2 to 3 hours, the lightly gelled material was transferred to dialysis tubing and dialysed extensively against water. Then it was lyophilized.

The crusty yellow solid was pulverized to a fine powder and dispersed in 10% gelatin to a concentration of 1%. A coating was made (170 µ), and dried at room temperature.

The film was evaluated with the following test mixture:

	Final Concentration
lithium lactate	250 mM
potassium phosphate buffer, pH 7.5	250 mM
glutathione	0.5 mM
glutathione reductase	9 U/mL
NAD	0.2 mM
LDH	5 U/mL

When contacted with this solution, the film produced a clear yellow color within one minute. A control solution, without LDH, produced no color.

The conclusion is that immobilizing the thiol reagent allows assay with thiol reagent sensitive enzymes.

The overall conclusion is that although many enzymes, such as alcohol dehydrogenase and cholesterol dehydrogenase are inhibited by DTNB according to the literature, there are many ways of avoiding this inhibition. Consequently, the second catalytic system which involves thiol indicators is generally applicable to assay of NADH as an intermediate and could be used to determine alcohol or cholesterol as analytes.

Example 5: Diffusible/Nondiffusible Dye Reaction

Another approach to generating a RAINBOW is where the hue of the test composition is made to appear as a result of diffusion. For instance, if an indicator is unable to diffuse due to being covalently linked to a matrix, and this matrix is covered with an opaque layer, then the indicator will be invisible from the top. If, as a result of a reaction, the covalent linkage of the indicator to the matrix is severed, then the indicator can diffuse up through the opaque covering and become visible.

As an example, a film was prepared as described in Example 4B, where DTNB was covalently bound to albumin which was then incorporated into a gelatin matrix. Whatman 31 ET was impregnated with a solution containing:

	Final Concentration
potassium phosphate, pH 7.5	160 mM
glutathione	0.4 mM
gluthathione reductase	36 U/ML

The impregnated paper was dried at 50 °C. Pieces of the dried paper were cut and laid on the gelatin film. The paper was quite opaque. An NADH solution was added to the multilayer device prepared in this way and water to another device to be used as a control. Within approximately 20 seconds, the device

contacted with NADH began to turn yellow and rapidly developed to a deep yellow. The control pad had no color.

An LDH assay was made with the following solution:

	Final Concentration
potassium phosphate, pH 7.5	250 mM
lithium lactate	250 mM
NAD	0.2 mM

LDH (about 5 U/mL) was added to this solution and 30 µL of the solution was added to one of the multilayer paper/gelatin device described above.

After about 5 1/2 minutes, the LDH pad showed a faint but definite yellow color. The control, without LDH, had no color.

The conclusion is that indicators can indeed be made diffusible as a function of analyte concentration.

In this approach, the indicator molecule is a combination of a hue determining (chromophoric) part and reactive (cleavable) part. These can be electronically isolated such that cleavage of the anchoring linkage does not significantly change the hue of the indicator. The amount, or intensity, of the indicator can be controlled by the activity of the catalytic system involved in the reductive cleavage and the hue which is generated is determined independently by the chromophoric part of the molecule. This is a considerable advantage as it can be difficult to find indicators with a suitable combination of hue and reactivity to generate the desired final hue.

Other matrices to which a thiol indicator could be immobilized by oxidation are: thiolagarose or any protein using a bifunctional reagent e.g., Lomant's reagent, dithiobis(succinimidyl propionate).

Claims

1. A test composition for the visual determination of an analyte in a fluid sample, comprising:
   (a) a catalytic system capable of generating reduced nicotinamide adenine dinucleotide from the analyte;
   (b) a first independent catalytic system capable of generating a reduced first indicator by reaction with reduced nicotinamide adenine dinucleotide; and
   (c) a second independent catalytic system capable of generating a change in hue of a second indicator component by reaction with reduced nicotinamide adenine dinucleotide, which second independent catalytic system includes:
      (i) a disulfide reductase;
      (ii) a disulfide substrate; and
      (iii) a thiol indicator; wherein the disulfide reductase is capable of catalyzing the reaction between reduced nicotinamide adenine dinucleotide and the disulfide substrate to produce a product which can interact with the thiol indicator to produce the change in hue of the second indicator;

   whereby the generation of the reduced first indicator and the changed hue of the second indicator occur substantially simultaneously to provide different hues at different analyte concentrations the particular final hue produced by the test composition depending on the concentration of the analyte.

2. A test composition according to claim 1 wherein the second independent catalytic system capable of generating a reduced second indicator by reaction with reduced nicotinamide adenine dinucleotide, which second independent catalytic system includes
   (i) a disulfide reductase;
   (ii) a disulfide substrate; and
   (iii) a thiol indicator; wherein the disulfide reductase is capable of catalyzing the reaction between reduced nicotinamide adenine dinucleotide and the disulfide substrate to produce a product which can reduce the thiol indicator to produce the reduced second indicator;

   whereby the generation of the reduced indicators occur substantially simultaneously to provide different hues at different analyte concentrations, the particular final hue produced by the test composition depending on the concentration of the analyte.

3. The test composition of any of claims 1 to 2 in which the first or second independent catalytic system is capable of producing a reduced third indicator.

4. The test composition of any of claims 1 to 3 in which one indicator component reduction produces a change from a hue to colorless.

5. The test composition of any of claims 1 to 4 which additionally include a color retardant.

6. The test composition of any of claims 1 to 5 in which the generation of the final hue of the test composition is essentially complete in less than about ten minutes.

7. The test composition of any of claims 1 to 6 in which the reduced second indicator is yellow.

8. The test composition of any of claims 1 to 7 in which the thiol indicator is a disulfide.

9. A test composition for the visual determination of glucose in an aqueous fluid sample, comprising:
(a) a catalytic system capable of generating reduced nicotinamide adenine dinucleotide by reaction with glucose;
(b) a first catalytic system composed of a catalyst having diaphorase activity, an oxidized first indicator and an oxidized third indicator each capable of reacting with reduced nicotinamide adenine dinucleotide in the presence of the catalyst to produce a reduced first and third indicator; and
(c) a second catalytic system composed of a disulfide reductase, a disulfide substrate and a thiol indicator, wherein the disulfide reductase is capable of catalyzing the reaction between reduced nicotinamide adenine dinucleotide and the disulfide substrate to produce a product which reduces the thiol indicator to produce a reduced second indicator;
whereby the generation of the reduced indicators occur substantially simultaneously to provide different hues at different analyte concentrations, the final hue produced by the test composition depending on the concentration of glucose in the sample.

10. The test composition of claim 9 in which the reduction of at least one of the first, third or thiol indicator produces a change from a hue to colorless.

11. A test device for the visual determination of an analyte in a fluid sample, comprising:
(a) a carrier matrix; and
(b) a test composition incorporated therewith, the test composition including: a catalytic system capable of generating reduced nicotinamide adenine dinucleotide from the analyte; a first independent catalytic system capable of generating a reduced first indicator by reaction with reduced nicotinamide adenine dinucleotide; and a second independent catalytic system capable of generating a yellow second indicator by reaction with reduced nicotinamide adenine dinucleotide, which second independent catalytic system includes
(i) a disulfide reductase;
(ii) a disulfide substrate; and
(iii) a thiol indicator; wherein the disulfide reductase is capable of catalyzing the reaction between reduced nicotinamide adenine dinucleotide and the disulfide substrate to produce a product which can interact with the thiol indicator to produce a yellow second indicator;
whereby the generation of the reduced and yellow indicators occur substantially simultaneously to provide different hues at different analyte concentrations, the particular final hue produced by the test composition depending on the concentration of the analyte.

12. The test device of claim 11 in which the first or second independent catalytic system is capable of producing a reduced third indicator.

13. The test device of any of claims 11 to 12 in which the reduction of at least one of the first or third oxidized indicators produced a change in the hue of that indicator of from a hue to colorless.

14. The test device of any of claims 11 to 13 in which at least one of the oxidized indicators or the thiol indicator is compartmentalized within the carrier matrix.

15. A multilayer test device for the visual determination of glucose in a body fluid sample,
(a) an inert support member, and in laminar relationship thereto;

(b) at least two layers composed of a hydrophilic carrier, one layer containing one of a first or third oxidized indicator component and the second layer containing

(i) nicotinamide adenine dinucleotide,

(ii) a glucose dehydrogenase capable of generating reduced nicotinamide adenine dinucleotide by reaction with glucose,

(iii) a disulfide substrate,

(iv) the other of the oxidized first indicator or the oxidized third indicator,

(v) a catalyst having diaphorase activity capable of facilitating the reaction between the reduced form of nicotinamide adenine dinucleotide and the oxidized first and third indicators to produce reduced first and third indicators,

(vi) a disulfide reductase; and

(vii) a thiol indicator; wherein the disulfide reductase is capable of catalyzing the reaction between the generated reduced nicotinamide adenine dinucleotide and the disulfide substrate to produce a product which can reduce the thiol indicator;

whereby the device is capable of generating substantially simultaneously the reduction of oxidized indicators, the particular final hue produced in the test device depending on the concentration of glucose in the sample.

16. The multilayer test device of claim 15 in which the hydrophilic carrier is gelatin and each layer is affixed by coating.

17. The multilayer test device of any of claims 15 to 16 in which the one layer additionally includes a color retardant.

Patentansprüche

1. Testzusammensetzung zur visuellen Bestimmung eines Analyten in einer flüssigen Probe, umfassend:

(a) ein katalytisches System, das zur Erzeugung von reduziertem Nicotinamid-adenin-dinucleotid aus dem Analyten in der Lage ist;

(b) ein erstes unabhängiges katalytisches System, das zur Erzeugung eines reduzierten ersten Indikators durch Umsetzung mit reduziertem Nicotinamid-adenin-dinucleotid in der Lage ist; und

(c) ein zweites unabhängiges katalytisches System, das zur Erzeugung einer Farbtonänderung einer zweiten Indikatorkomponente durch Umsetzung mit reduziertem Nicotinamid-adenin-dinucleotid in der Lage ist, wobei das zweite unabhängige katalytische System folgende Bestandteile umfaßt:

(i) eine Disulfid-reduktase;

(ii) ein Disulfid-Substrat; und

(iii) einen Thiol-Indikator; wobei die Disulfid-reduktase in der Lage ist, die Reaktion zwischen reduziertem Nicotinamid-adenin-dinucleotid und dem Disulfid-Substrat unter Bildung eines Produkts zu katalysieren, das mit dem Thiol-Indikator unter Erzeugung einer Farbtonänderung des zweiten Indikators in Wechselwirkung treten kann;

wobei die Erzeugung des reduzierten ersten Indikators und der veränderte Farbton des zweiten Indikators im wesentlichen gleichzeitig erfolgen, so daß unterschiedliche Farbtöne bei unterschiedlichen Analytkonzentrationen hervorgerufen werden, wobei der von der Testzusammensetzung erzeugte endgültige Farbton von der Konzentration des Analyten abhängt.

2. Testzusammensetzung nach Anspruch 1, wobei das zweite unabhängige katalytische System, das durch Umsetzung mit reduziertem Nicotinamid-adenin-dinucleotid zur Erzeugung eines reduzierten zweiten Indikators in der Lage ist, folgende Bestandteile umfaßt:

(i) eine Disulfid-reduktase;

(ii) ein Disulfid-Substrat; und

(iii) einen Thiol-Indikator; wobei die Disulfid-reduktase in der Lage ist, die Reaktion zwischen reduziertem Nicotinamid-adenin-dinucleotid und dem Disulfid-Substrat unter Bildung eines Produkts zu katalysieren, das den Thiol-Indikator unter Bildung des reduzierten zweiten Indikators reduzieren kann;

wobei die Erzeugung der reduzierten Indikatoren im wesentlichen gleichzeitig erfolgt, so daß sich bei unterschiedlichen Analytkonzentrationen unterschiedliche Farbtöne ergeben, wobei der durch die Testzusammensetzung erzeugte endgültige Farbton von der Analytkonzentration abhängt.

3. Testzusammensetzung nach einem der Ansprüche 1 oder 2, in der das erste oder zweite unabhängige katalytische System zur Bildung eines reduzierten dritten Indikators in der Lage ist.

4. Testzusammensetzung nach einem der Ansprüche 1 bis 3, in der die Reduktion einer Indikatorkomponente eine Veränderung eines Farbtons zu einer farblosen Beschaffenheit erzeugt.

5. Testzusammensetzung nach einem der Ansprüche 1 bis 4, die zusätzlich ein Farbverzögerungsmittel enthält.

6. Testzusammensetzung nach einem der Ansprüche 1 bis 5, in der die Erzeugung des endgültigen Farbtons der Testzusammensetzung innerhalb von weniger als etwa 10 Minuten im wesentlichen vollständig ist.

7. Testzusammensetzung nach einem der Ansprüche 1 bis 6, in der der reduzierte zweite Indikator gelb ist.

8. Testzusammensetzung nach einem der Ansprüche 1 bis 7, in der es sich bei dem Thiol-Indikator um ein Disulfid handelt.

9. Testzusammensetzung zur visuellen Bestimmung von Glucose in einer wäßrigen flüssigen Probe, enthaltend:
(a) ein katalytisches System, das zur Erzeugung von reduziertem Nicotinamid-adenin-dinucleotid durch Reaktion mit Glucose in der Lage ist,
(b) ein erstes katalytisches System, das aus einem Katalysator mit Diaphorase-Aktivität, einem oxidierten ersten Indikator und einem oxidierten dritten Indikator zusammengesetzt ist, die jeweils in der Lage sind, mit reduziertem Nicotinamid-adenin-dinucleotid in Gegenwart des Katalysators unter Bildung des reduzierten ersten und dritten Indikators zu reagieren; und
(c) ein zweites katalytisches System, das aus einer Disulfid-reduktase, einem Disulfid-Substrat und einem Thiol-Indikator zusammengesetzt ist, wobei die Disulfid-reduktase in der Lage ist, die Reaktion zwischen reduziertem Nicotinamid-adenin-dinucleotid und dem Disulfid-Substrat zu katalysieren, wodurch ein Produkt gebildet wird, das den Thiol-Indikator unter Bildung eines reduzierten zweiten Indikators reduziert;
wobei die Erzeugung der reduzierten Indikatoren im wesentlichen gleichzeitig erfolgt, wodurch bei unterschiedlichen Analytkonzentrationen unterschiedliche Farbtöne entstehen, wobei der durch die Testzusammensetzung gebildete endgültige Farbton von der Glucosekonzentration in der Probe abhängt.

10. Testzusammensetzung nach Anspruch 9, in der die Reduktion von mindestens einem Indikator aus der Gruppe des ersten, dritten oder Thiol-Indikators eine Änderung von einem Farbton zu einer farblosen Beschaffenheit erzeugt.

11. Testvorrichtung zur visuellen Bestimmung eines Analyten in einer flüssigen Probe, enthaltend:
(a) eine Trägermatrix; und
(b) eine darin einverleibte Testzusammensetzung, die folgendes umfaßt: ein katalytisches System, das zur Erzeugung von reduziertem Nicotinamid-adenin-dinucleotid aus dem Analyten in der Lage ist; ein erstes unabhängiges katalytisches System, das zur Erzeugung eines reduzierten ersten Indikators durch Umsetzung mit reduziertem Nicotinamid-adenin-dinucleotid in der Lage ist; und ein zweites unabhängiges katalytisches System, das zur Erzeugung eines gelben zweiten Indikators durch Umsetzung mit reduziertem Nicotinamid-adenin-dinucleotid in der Lage ist, wobei das zweite unabhängige katalytische System folgendes umfaßt:
(i) eine Disulfid-reduktase;
(ii) ein Disulfid-Substrat; und
(iii) einen Thiol-Indikator; wobei die Disulfid-reduktase in der Lage ist, die Reaktion zwischen reduziertem Nicotinamid-adenin-dinucleotid und dem Disulfid-Substrat unter Bildung eines Produkts zu katalysieren, das mit dem Thiol-Indikator unter Erzeugung einer Farbtonänderung des zweiten Indikators in Wechselwirkung treten kann;
wobei die Erzeugung der reduzierten und gelben Indikatoren im wesentlichen gleichzeitig erfolgt, wodurch unterschiedliche Farbtöne bei unterschiedlichen Analytkonzentrationen gebildet werden,

wobei der durch die Testzusammensetzung erzeugte spezielle endgültige Farbton von der Konzentration des Analyten abhängt.

12. Testvorrichtung nach Anspruch 11, wobei das erste oder zweite unabhängige katalytische System zur Erzeugung eines reduzierten dritten Indikators in der Lage ist.

13. Testvorrichtung nach einem der Ansprüche 11 oder 12, in der die Reduktion des ersten und/oder dritten oxidierten Indikators eine Farbtonänderung des Indikators von einem Farbton zu einer farblosen Beschaffenheit erzeugt.

14. Testvorrichtung nach einem der Ansprüche 11 bis 13, in der mindestens einer der oxidierten Indikatoren oder des Thiol-Indikators innerhalb der Trägermatrix kompartimentiert ist.

15. Mehrschichtige Testvorrichtung zur visuellen Bestimmung von Glucose in einer Körperflüssigkeitsprobe, umfassend
(a) ein inertes Trägerelement und in laminarer Beziehung dazu;
(b) mindestens zwei Schichten, zusammengesetzt aus einem hydrophilen Träger, wobei eine Schicht einen Bestandteil aus einer ersten oder dritten oxidierten Indikatorkomponente enthält und die zweite Schicht folgendes enthält:
(i) Nicotinamid-adenin-dinucleotid,
(ii) eine Glucose-dehydrogenase, die zur Erzeugung von reduziertem Nicotinamid-adenin-dinucleotid durch Umsetzung mit Glucose in der Lage ist;
(iii) ein Disulfid-Substrat,
(iv) den anderen Bestandteil aus der Gruppe oxidierter erster Indikator und oxidierter dritter Indikator,
(v) einen Katalysator mit Diaphorase-Aktivität, der dazu in der Lage ist, die Reaktion zwischen der reduzierten Form von Nicotinamid-adenin-dinucleotid und dem oxidierten ersten und dritten Indikator unter Bildung der reduzierten ersten und dritten Indikatoren zu katalysieren,
(vi) eine Disulfid-reduktase; und
(vii) einen Thiol-Indikator; wobei die Disulfid-reduktase in der Lage ist, die Reaktion zwischen dem erzeugten reduzierten Nicotinamidadenin-dinucleotid und dem Disulfid-Substrat unter Bildung eines Produkts, das den Thiol-Indikator reduzieren kann, zu katalysieren;
wobei die Vorrichtung dazu in der Lage ist, die Reduktion der oxidierten Indikatoren im wesentlichen gleichzeitig ablaufen zu lassen, wobei der in der Testvorrichtung gebildete spezielle endgültige Farbton von der Glucosekonzentration in der Probe abhängt.

16. Mehrschichtige Testvorrichtung nach Anspruch 15, in der es sich beim hydrophilen Träger um Gelatine handelt und die einzelnen Schichten durch Beschichtung aufgebracht sind.

17. Mehrschichtige Testvorrichtung nach einem der Ansprüche 15 bis 16, in der die eine Schicht zusätzlich ein Farbverzögerungsmittel enthält.

Revendications

1. Composition d'essai pour la détermination à l'oeil nu d'un analyte dans un échantillon de fluide, comprenant :
(a) un système catalytique capable de générer du nicotinamide-adénine-dinucléotide de forme réduite, à partir de l'analyte;
(b) un premier système catalytique indépendant capable de générer un premier indicateur de forme réduite par la mise en réaction avec du nicotinamide-adénine-dinucléotide de forme réduite; et
(c) un second système catalytique indépendant capable de générer un changement de teinte d'un second composant d'indicateur par réaction avec du nicotinamide-adénine-dinucléotide de forme réduite, ledit second système catalytique indépendant englobant :
(i) une disulfure-réductase,
(ii) un substrat de disulfure, et
(iii)un indicateur de thiol; dans lequel la disulfure-réductase est capable de catalyser la réaction entre le nicotinamide-adénine-dinucléotide de forme réduite et le substrat de disulfure pour procurer un produit qui peut interagir avec l'indicateur de thiol pour procurer le changement de

teinte du second indicateur;
par laquelle la génération du premier indicateur de forme réduite et la modification de teinte du second indicateur apparaissent de manière essentiellement simultanée pour procurer différentes teintes à différentes concentrations d'analyte, la teinte finale particulière produite par la composition d'essai dépendant de la concentration de l'analyte.

2. Composition d'essai selon la revendication 1, dans laquelle le second système catalytique indépendant est capable de générer un second indicateur de forme réduite par réaction avec du nicotinamide-adénine-dinucléotide de forme réduite, ledit second système catalytique indépendant englobant :
(i) une disulfure-réductase,
(ii) un substrat de disulfure, et
(iii)un indicateur de thiol; dans lequel la disulfure-réductase est capable de catalyser la réaction entre le nicotinamide-adénine-dinucléotide de forme réduite et le substrat de disulfure pour procurer un produit qui peut réduire l'indicateur de thiol pour procurer le second indicateur de forme réduite;
par laquelle la génération des indicateurs de forme réduite apparaît de manière essentiellement simultanée pour procurer différentes teintes à différentes concentrations d'analyte, la teinte finale particulière produite par la composition d'essai dépendant de la concentration de l'analyte.

3. Composition d'essai selon l'une quelconque des revendications 1 ou 2, dans laquelle le premier ou le second système catalytique indépendant est capable de produire un troisième indicateur de forme réduite.

4. Composition d'essai selon l'une quelconque des revendications 1 à 3, dans laquelle une réduction de composant d'indicateur procure un changement par lequel on passe d'une teinte à l'état incolore.

5. Composition d'essai selon l'une quelconque des revendications 1 à 4, qui englobe, en outre, un retardateur des couleurs.

6. Composition d'essai selon l'une quelconque des revendications 1 à 5, dans laquelle la génération de la teinte finale de la composition d'essai arrive essentiellement à son terme en un laps de temps inférieur à environ 10 minutes.

7. Composition d'essai selon l'une quelconque des revendications 1 à 6, dans laquelle le second indicateur de forme réduite est jaune.

8. Composition d'essai selon l'une quelconque des revendications 1 à 7, dans laquelle l'indicateur de thiol est un disulfure.

9. Composition d'essai pour la détermination à l'oeil nu de la teneur en glucose dans un échantillon de fluide aqueux, comprenant :
(a) un système catalytique capable de générer du nicotinamide-adénine-dinucléotide de forme réduite par réaction avec du glucose;
(b) un premier système catalytique composé d'un catalyseur exerçant une activité de diaphorase, d'un premier indicateur de forme oxydée et d'un troisième indicateur de forme oxydée, chacun étant capable de réagir avec du nicotinamide-adénine-dinucléotide de forme réduite en présence du catalyseur pour produire un premier et un troisième indicateurs de forme réduite, et
(c) un second système catalytique composé d'une disulfure-réductase, d'un substrat de disulfure et d'un indicateur de thiol, dans laquelle la disulfure-réductase est capable de catalyser la réaction entre le nicotinamide-adénine-dinucléotide de forme réduite et le substrat de disulfure pour procurer un produit qui réduit l'indicateur de thiol pour produire un second indicateur de forme réduite;
par laquelle la génération des indicateurs de forme réduite apparaît de manière essentiellement simultanée pour procurer différentes teintes à différentes concentrations d'analyte, la teinte finale produite par la composition d'essai dépendant de la concentration de glucose dans l'échantillon.

10. Composition d'essai selon la revendication 9, dans laquelle la réduction d'au moins un indicateur parmi le premier, le troisième ou l'indicateur de thiol produit un changement par lequel on passe d'une teinte à l'état incolore.

11. Dispositif d'essai pour la détermination à l'oeil nu d'un analyte dans un échantillon de fluide, comprenant :
   (a) une matrice de support, et
   (b) une composition d'essai incorporée dans cette dernière, la composition d'essai englobant : un système catalytique capable de générer du nicotinamide-adénine-dinucléotide de forme réduite, à partir de l'analyte; un premier système catalytique indépendant capable de générer un premier indicateur de forme réduite par la mise en réaction avec du nicotinamide-adénine-dinucléotide de forme réduite; et un second système catalytique indépendant capable de générer un second indicateur jaune par réaction avec du nicotinamide-adénine-dinucléotide de forme réduite, ledit second système catalytique indépendant englobant
      (i) une disulfure-réductase,
      (ii) un substrat de disulfure, et
      (iii)un indicateur de thiol; dans lequel la disulfure-réductase est capable de catalyser la réaction entre du nicotinamide-adénine-dinucléotide de forme réduite et le substrat de disulfure pour procurer un produit qui peut interagir avec l'indicateur de thiol pour procurer un second indicateur jaune;

   par lequel la génération des indicateurs jaune et de forme réduite apparaît de manière essentiellement simultanée pour procurer différentes teintes à différentes concentrations d'analyte, la teinte finale particulière produite par la composition d'essai dépendant de la concentration de l'analyte.

12. Dispositif d'essai selon la revendication 11, dans lequel le premier ou le second système catalytique indépendant est capable de produire un troisième indicateur de forme réduite.

13. Dispositif d'essai selon l'une quelconque des revendications 11 ou 12, dans lequel la réduction d'au moins un indicateur parmi le premier ou le troisième indicateur de forme oxydée produit un changement de la teinte de cet indicateur, par lequel on passe d'une teinte à l'état incolore.

14. Dispositif d'essai selon l'une quelconque des revendications 11 à 13, dans lequel au moins un des indicateurs de forme oxydée ou l'indicateur de thiol est compartimenté au sein de la matrice de support.

15. Dispositif d'essai multicouche pour la détermination à l'oeil nu de la teneur en glucose dans un échantillon de liquide organique, comprenant
   (a) un élément de support inerte et, en relation laminaire avec ce dernier,
   (b) au moins deux couches composées d'un support hydrophile, une couche contenant un premier ou un troisième composant d'indicateur de forme oxydée et la seconde couche contenant
      (i) du nicotinamide-adénine-dinucléotide,
      (ii) une glucose-déshydrogénase capable de générer du nicotinamide-adénine-dinucléotide de forme réduite par réaction avec du glucose,
      (iii)un substrat de disulfure,
      (iv) l'autre indicateur parmi le premier indicateur de forme oxydée ou le troisième indicateur de forme oxydée,
      (v) un catalyseur exerçant une activité de diaphorase capable de faciliter la réaction entre la forme réduite du nicotinamide-adénine-dinucléotide et les premier et troisième indicateurs de forme oxydée pour produire des premier et troisième indicateurs de forme réduite,
      (vi) une disulfure-réductase, et
      (vii)un indicateur de thiol; dans lequel la disulfure-réductase est capable de catalyser la réaction entre le nicotinamide-adénine-dinucléotide de forme réduite, qui a été généré, et le substrat de disulfure pour procurer un produit qui peut réduire l'indicateur de thiol;

   par lequel le dispositif est capable de générer de manière essentiellement simultanée la réduction des indicateurs de forme oxydée, la teinte finale particulière produite par la composition d'essai dépendant de la concentration de l'analyte.

16. Dispositif d'essai multicouche selon la revendication 15, dans lequel le support hydrophile est de la gélatine et chaque couche est fixée par enduction.

17. Dispositif d'essai multicouche selon l'une quelconque des revendications 15 ou 16, dans lequel la première couche englobe, en outre, un retardateur des couleurs.